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SUITABLE HOST FISH, POPULATION STRUCTURE, AND LIFE-HISTORY CHARACTERISTICS FOR THE STATE-LISTED, LOUISIANA PIGTOE, PLEUROBEMA RIDDELLII

Elizabeth Hinkle
University of Texas at Tyler

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by

ELIZABETH HINKLE

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ELIZABETH G HINKLE

has been approved for the thesis requirements on
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for the Master of Science degree

Approvals:

Thesis/Dissertation Chair: Neil Ford, Ph.D.

Member: Lance Williams, Ph.D.

Member: John S. Placyk, Jr., Ph.D.

Chair, Department of Biology

Dean, College of Arts and Sciences

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Abstract

SUITABLE HOST FISH, POPULATION STRUCTURE, AND LIFE HISTORY CHARACTERISTICS FOR THE STATE-LISTED, LOUISIANA PIGTOE, *PLEUROBEMA RIDDELLII*

Elizabeth G. Hinkle

Thesis Chair: Neil Ford, Ph.D.

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There are 53 extant Unionids in Texas, six of which are petitioned for federal protection under the Endangered Species Act of 1973. The Louisiana Pigtoe, *Pleurobema riddellii*, has recorded distribution in Texas, Arkansas, and Louisiana. In Texas *P. riddellii* historically occurs as far west as the San Jacinto and Trinity rivers, eastward to the Neches, Sabine and Red rivers. Its abundance is low, and an understanding of their life-history and reproductive characteristics is imperative for establishing federal protection status. During the larval stage of unionid mussels' life cycle, juveniles are obligate parasites of fish. This study sought to determine the host fish for *P. riddellii* by collecting wild infected fish from the upper Neches River, and observing each species separately in the laboratory. Juvenile mussels that metamorphosed from the fish were preserved, cataloged, and analyzed genetically. This research indicated the blacktail shiner *(Cyprinella venusta)* was a host-fish of *P. riddellii* glochidia, supporting claims that *P. riddellii* are Cyprinid specialists. Data also characterize adult spawning and juvenile abundance throughout the season. Additionally, I collected gonadal fluid samples of *P. riddellii* throughout the year to provide insight into population structure, reproductive size, fecundity potential, and spawning. Analyses indicate that *P. riddellii* exhibit an equilibrium life-history strategy of low to moderate growth rates, and low but variable fecundity throughout their life.

Chapter 1: Determining Suitable Host-fish of *Pleurobema riddellii*

Background

There are an estimated 1,000 species of freshwater bivalves worldwide with the freshwater mussel order Unionoida accounting for about 850 of those species (Haszprunar et al., 2008). Of that 850, 300 occur in North America, making it the most speciose continent (Graf & Cummings, 2007). Bivalves often compose the largest benthic biomass in rivers, and early naturalists report being unable to wade across rivers without treading on mussels (Simpson, 1899). Now, 78 of the North American species are listed as endangered, threatened or of special concern (U.S. Fish and Wildlife Service, 2016). Furthermore, it is currently projected that North America stands to lose up to half of its freshwater mussel species within the next 100 years (Ricciardi & Rasmussen, 1999). This decline in mussel biodiversity and abundance has spurred research nationwide, focusing largely on the ecology and life-history of endangered species. The Louisiana Pigtoe, *Pleurobema riddellii,* is one of 53 native Texas mussels. It has been documented or believed to potentially occur in 7 Arkansas counties, 5 Louisiana parishes, and 51 Texas counties (Howells, 1997; Howells et al., 1996; Vidrine, 1993). This species historically occurs in Texas in the San Jacinto, Trinity, Neches-Angelina, Sabine, Big Cypress, and Sulfur River basins (Howells, 1997; Howells et al., 1996; Vidrine, 1993). *Pleurobema riddellii* is currently state-listed and is one of six Texas species being petitioned for protection under the Endangered Species Act of 1973 (ESA) (Winemiller et al., 2010). For a species to be eligible for federal protection, life-history information must be available including reproductive ecology.

Unionid mussels have developed unique reproductive mechanisms adapted to their mostly sedentary life cycle. During spawning, males release their sperm into the water, and the females intake the sperm through their incurrent siphon. Simultaneously, the female releases eggs from the gonads into the suprabranchial chamber, dorsal to the gills, which is the likely site of fertilization. While the eggs are ultimately fertilized internally they are not fertilized within the reproductive tract, therefore Unionids are classified as spermcasters (Haggerty et al., 1995; Yokley, 1972). Upon fertilization, the eggs are brooded within the female until they develop into their larval state, called glochidia. Glochidia are then released from the female's suprabranchial chamber into the water column where they must attach themselves to a host fish and encyst onto the gills or exposed flesh (Haag, 2012; Heard & Dinesen, 1999; Yokley, 1972). There are two species of unionids that have been documented as having developed the secondary ability to bypass this parasitic stage, but both species still have ability to parasitize onto fish. Other than these two species, all unionid glochidia are obligate parasites (Barfield & Watters, 1998; Dickinson & Sietman, 2008; Lellis & King, 1998). Some unionid glochidia have developed features such as hooks, jagged teeth, and sensory hairs which allow them to grip onto the host fish (Haag, 2012).

Glochidia attach along the esophagus, inside the operculum, around the nostrils and mouth or the fins of host fish. Glochidia can remain in their parasitic stage for several weeks until they detach as fully metamorphosed juvenile mussels (Haag, 2012). Glochidia can only develop into juveniles on host fish that have not developed an immune response to prevent the parasitism (Kirk & Layzer, 1997; Meyers et al.,

1980; O'Connell & Neves, 1999; Waller & Mitchell, 1989). After a succession of infections, an enzymatic response of host fish can lead to glochidia being rejected. Of the 130 mussel species with host information, approximately 80 percent are specialized to a specific host fish, while the remaining about 20 percent are generalists (Cummings & Watters, 2010). It is anticipated that *P. riddellii* may be a specialist given the general trend seen in unionids and because other *Pleurobema* that have been studied have been specialists (Bertramet al., 2017; Layzer et al., 2003; Weaver et al., 1991).

It is postulated that mussels in the genus *Pleurobema* release glochidia via pelagic conglutinates (gelatinous clumps of fertilized eggs which float through the water), that typically attach to drift feeding minnows (Haag, 2012; Hove & Neves, 1994). Two fish in the *Cyprinidae* family, *Cyprinella lutrensis* (Red Shiner), and *Pimephales vigilax* (Bullhead Minnow) have been reported as potential hosts (Bertram et al., 2018; Marshall et al., 2018). However, only five *P. riddellii* glochidia were identified throughout these studies being found encysted upon three *C. lutrensis*, and one *P. vigilax*. Each infected fish had less than three encysted glochidia (Marshall et al., 2018). Previously recorded *P. riddellii* encystments could be low because of an activated immune response of host fish. As fish are exposed to glochidia they may gain immunity to the parasitic juvenile, which can result in failed metamorphosis (Dodd et al., 2005; Rogers-Lowery & Dimock Jr, 2006). Additionally, Marshall et al. (2018) collected glochidia that were still encysted, so could not account for the full metamorphosis from glochidia to juvenile. Therefore, our confidence in these fishes as hosts for *P. riddellii* needs to remain somewhat skeptical. The current study seeks to establish a more robust link between *P. riddellii* and their potential host fish, and thus

suggest fish that need to be included in conservation efforts if *Pleurobema riddellii* is federally listed.

In general, the laboratory setting does not provide as accurate of data on host fish suitability as natural settings do (Österling & Söderberg, 2015). For example, in the laboratory glochidia can encyst upon a variety of ages of fish, whereas in the wild, only younger fish served as hosts. Mussels can also infect fish in the laboratory, even though that species of fish did not exist within the mussel's natural stream system (Täubert, 2014). The technique of testing natural infestation is more time and cost efficient (Österling & Wengström, 2015). Thus, to employ the most efficient and accurate analysis of host identification, this study examined fully metamorphosed juveniles from fish infected in the wild.

It is suspected that flow rate may impact spawning times, and several studies indicate that temperature may be a primary component in inducing spawning (Galbraith & Vaughn, 2009; Hastie & Young, 2003; Holland-Bartels & Kammer, 1989; Neves et al., 1985). Therefore, during fish collection water temperature was taken, and flow rate (daily mean $feet^3$ per second), and depth (daily mean feet) for that date were obtained from the U.S. Geological Service from site number 08032000 located on the Neches River near Neches, Texas (Texas Water Data Support Team, 2018) and correlated with juvenile abundance. This investigation into host-specificity is important in understanding the species, and a necessary step in determining its federal protection, and potentially the protection of the host fish that *P. riddellii* are dependent.

Methods

Fish collection

Fish were collected eight separate times from March 2017 through October 2017, from two sites on the Neches River. This time frame was used because previous research indicated that mussels within the Neches River basin spawned from Spring into Fall (Marshall et al., 2018) We targeted mussel beds with relatively high abundance of *P. riddellii* and which were in the habitat most likely to find them (i.e., gravely runs) based on recent previous work in this system (Ford et al., 2014; Glen, 2017). These mussel beds were chosen to increase the likelihood of capturing fishes infected with this species. Each sampling sites was electroshocked with a Smith-Root SR-6 Tote Barge® and fish were collected with dip nets. Beach seining was also used when water and flow allowed for such technique. Sampling sites included the entire width of the river at approximately 50-meter segments. Upon collection, fish were placed into coolers with ice-packs (to prevent the fish from overheating) and battery powered aerators and transported to the Vivarium at the University of Texas at Tyler Biology Department.

Fish housing and juvenile collection

At the Vivarium fish were placed into an Aquatics Habitat unit (AHAB Pentair Aquatics®) in three-liter tanks segregated by species. Additionally, to accommodate larger fish, a second aquatic habitat was built with tanks which held sixteen-liter tanks. Each compartment within the AHABs housed between one and sixteen fish of each species in accordance with fish size, proper tank aeration, and social behavior (ie: some sunfishes can be territorial, so they were occasionally relocated to avoid stress). At the water expulsion point on the back of each compartment, a “juvenile catcher” was

attached. A juvenile catcher is a small segment of 3.5cm PVC pipe with 112-micron mesh stretched across its base to catch glochidia or juveniles as they fall from the fish (Bertram et al., 2017). This mesh size was chosen to accommodate the estimated glochidia and juvenile size range (Barnhart, 2006).

Catchers were manually checked daily by removing the catchers from the back of each compartment and rinsing them with deionized water into a Petri dish. Juveniles were examined under a dissection microscope to gauge their developmental stage. Juveniles had soft tissue development and exhibited darker coloration and the ability to snap their shells closed by using their muscular hinge, whereas glochidia did not have tissue development, appeared clear and were unable to snap their shells closed. All juveniles were preserved in 1.5 mL centrifuge tubes of 95 percent ethanol. The number of juveniles per each fish species was recorded to estimate severity of infection. Since closely related species parasitized upon their host fish for two to six weeks, we were also able to estimate the timing of infection (Culp et al., 2009; Hove & Neves, 1994). After at least four weeks in the AHABs the fish were euthanized via ice-bath and preserved for further analysis.

The fish were fed daily with TetraMin® food flakes. The water was monitored daily for nitrate, nitrite, ammonia, pH, dissolved oxygen, and temperature. Water quality was maintained using Prime®, Stability®, PhosGuard™, buffers, Puragin® pouches, and frequent water changes. The pH was kept between 6 and 7, and 10% of the water was changed weekly. Occasionally newly collected samples would lead to a spike in nitrate and ammonia levels, at which point the water was changed more

frequently. The temperature was maintained by the central air system in the laboratory (23-27°C).

Genetic Analysis

All juvenile mussels were placed in a 1.5mL centrifuge tube with of 95 percent ethanol and stored in a -20-degree freezer. Only juveniles were used in the genetic analysis because they represent successful metamorphosis, and thus verify a compatible host because the host's immune system did not reject them (Haag, 2012; Watters & O'Dee, 1996). Juveniles were distinguishable from glochidia by the presence of tissue development, movement, and the ability to quickly close their shells (Howells et al., 1996). Genomic DNA was extracted from each sample using a Cetyl trimethylammonium bromide (CTAB) DNA extraction protocol (Campbell et al., 2005; modified by Powell, 2018). Extracted DNA was analyzed on a Nanodrop spectrophotometer to verify presence of quality DNA. The DNA samples were then amplified through polymerase chain reaction (PCR) using a set of mussel-specific primers (Table 1) that targeted mitochondrial Cytochrome c Oxidase Subunit 1 (COI) (Campbell et al., 2005, modified from Folmer et al., 1994). An additional primer pair, Leu-uurF / NIJ-12073 (Serb et al., 2003) was used to amplify NADH Dehydrogenase Subunit 1 (ND1) (Serb et al., 2003) (Table 1).

All polymerase chain reactions were carried out at a volume of 25ul and included 0.1ul Invitrogen Platinum Taq DNA Polymerase, 0.75ul 50mM $MgCl_2$, 0.5ul 10mM dNTPs, 0.5ul forward primer, 0.5ul reverse primer, 2.5ul PCR buffer, 14.15ul nanopure water, and 6ul template DNA. For both NDI and COI polymerase chain reaction the following thermocycler protocol was used: 94°C for 5 min, 94°C for 45 secs, 54°C for 1

min, 72°C for 1 min for 35 cycles, then 72°C for 10 min. Gel electrophoresis on a 1-2% agarose gel was used to observe any amplicons from the PCR. The amplified DNA product was isolated by ethanol precipitation. Isolated DNA was eluted in water and sent to Eurofins Genomics for sequencing. Remaining DNA was stored in a -20 freezer for potential future use. The sequences were compared with sequences on the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov), and cross-referenced with the adult molecular sequences to determine species identities (Campbell & Lydeard, 2012).

Table 1. Polymerase chain reaction primers used for amplification and sequencing of the ND1 and COI mitochondrial genes in juvenile unionid mussels.

Gene	Primer	Sequence (5' - 3')	Source
ND1	Leu-uurF	5'-TGG CAG AAA AGT GCA TCA GAT TAA AGC-3'	Serb et al. 2003
ND1	NIJ-12073	5'-TCG GAA TTC TCC TTC TGC AAA GTC-3'	Serb et al. 2003
COI	LCO1490	5'-GTT CCA CAA ATC ATA AGG ATA TTG G-3'	Campbell et al. 2005
COI	HCO2198	5'-TAC ACC TCA GGG TGA CCA AAA AAC CA-3'	Campbell et al. 2005

Additional analyses

Peak juvenile abundance was monitored, as was fish species in association with juvenile abundance. A linear model was used to correlate the abundance of juveniles with lagged flow rate (mean daily cubic feet per second at 35 and 40 days prior to observing juveniles), and lagged depth (daily mean feet at 35and 40 days prior to observing juveniles) to understand the relationship of these variables with infection rates (Texas Water Data Support Team, 2018; R Core Team, 2013; Smith, N., personal communication, May 10, 2018). Because *P. riddellii* were estimated to parasitize fish for several weeks, the attempted models included lag times of 35, and 40 days prior so that I

would be observing the weather events when the fish first became infected. The fish that were in tanks, which housed juvenile mussels were measured post-euthanasia via ice bath. Lengths were measured to the nearest hundredth of a millimeter using calipers, and any additional encysted juveniles were collected for genetic analyses. These data were used to compare between average fish body size and juvenile infection rate.

Results

Four of the eight fish collection periods produced wild infected fish. Approximately 513 juvenile mussels were collected from the infected fishes between July 30 and October 31, 2017; 443 juveniles came from Blacktail Shiners (*Cyprinella venusta*), 39 from Red Shiners (*C. lutrensis*), 15 from Bullhead Minnows (*Pimephales vigilax*), 11 from weed shiners, (*Notropis texanus*) and 5 from Darters (genus *Etheostoma*). There was no relationship between the flow and depth at 35 nor 40 days prior to juveniles dropping from fish (p-values of flow and depth lagged forty days prior to juvenile presence, and thirty-five days prior were 0.7696 and 0.2343, respectively) (Figures 2 and 3). All individual juveniles that sequenced successfully were found in tanks which housed *C. venusta* (Table 3). The correlation between average fish body length and average juvenile infection rate was not strong (r^2=0.29) (XLSTAT, 2017).

Table 2. Fish collection dates, methods, and locations from sampling events in which juvenile mussels were collected.

Date Fish Collected	Juveniles Collected	Collection Method	Fishing Location	
21-Jul	29	Electrofish	31.64344, -95.28617	
9-Sep	59	Electrofish	31.64344, -95.28617	
16-Sep	11	Seine	31.63238, -95.28441	31.64344, -95.28617
8-Oct	414	Seine	31.63522, -95.28158	31.64344, -95.28617
	Total=513			

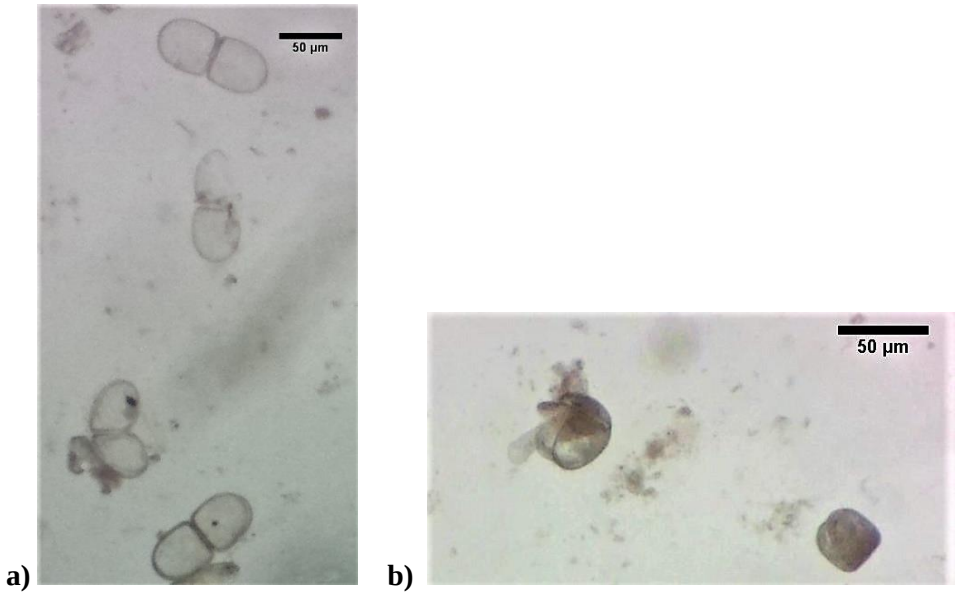


Figure 1. Photo a) four glochidia, which did not successfully metamorphose and are absent of muscle tissue. Photo b) two juveniles with developed soft tissue and a hinge which can quickly snap the shell closed.

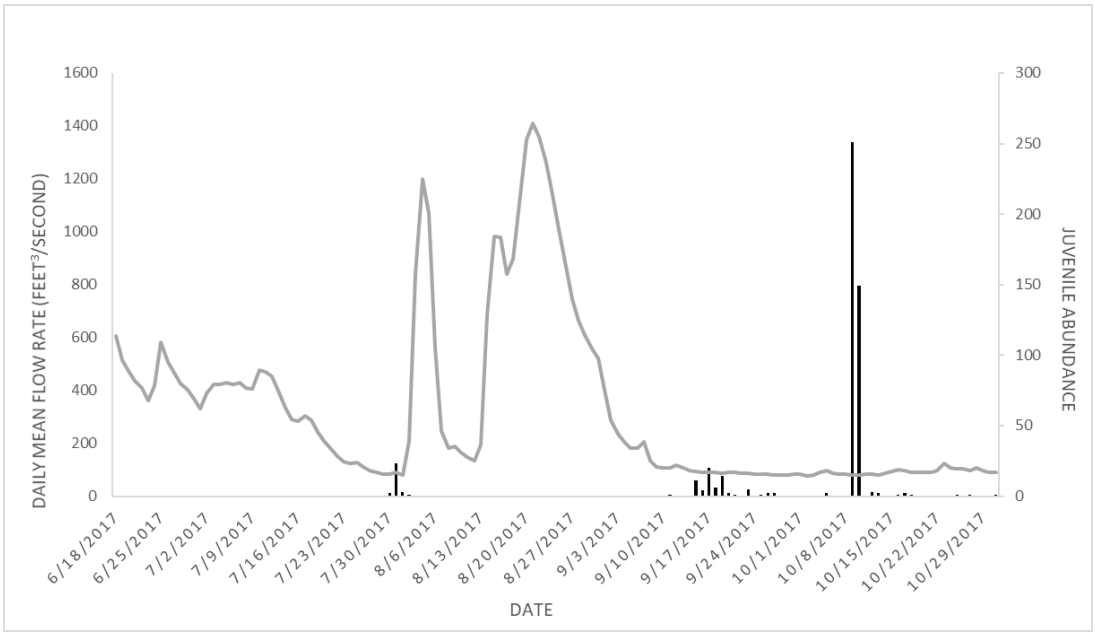


Figure 2. Relationship between flow rate (Texas Water Data Support Team, 2018) and juvenile abundance observed in lab. The y-axis on the left represents daily mean flow rate near the sampling site in the Neches River, and the second y-axis in the right indicates juvenile abundance.

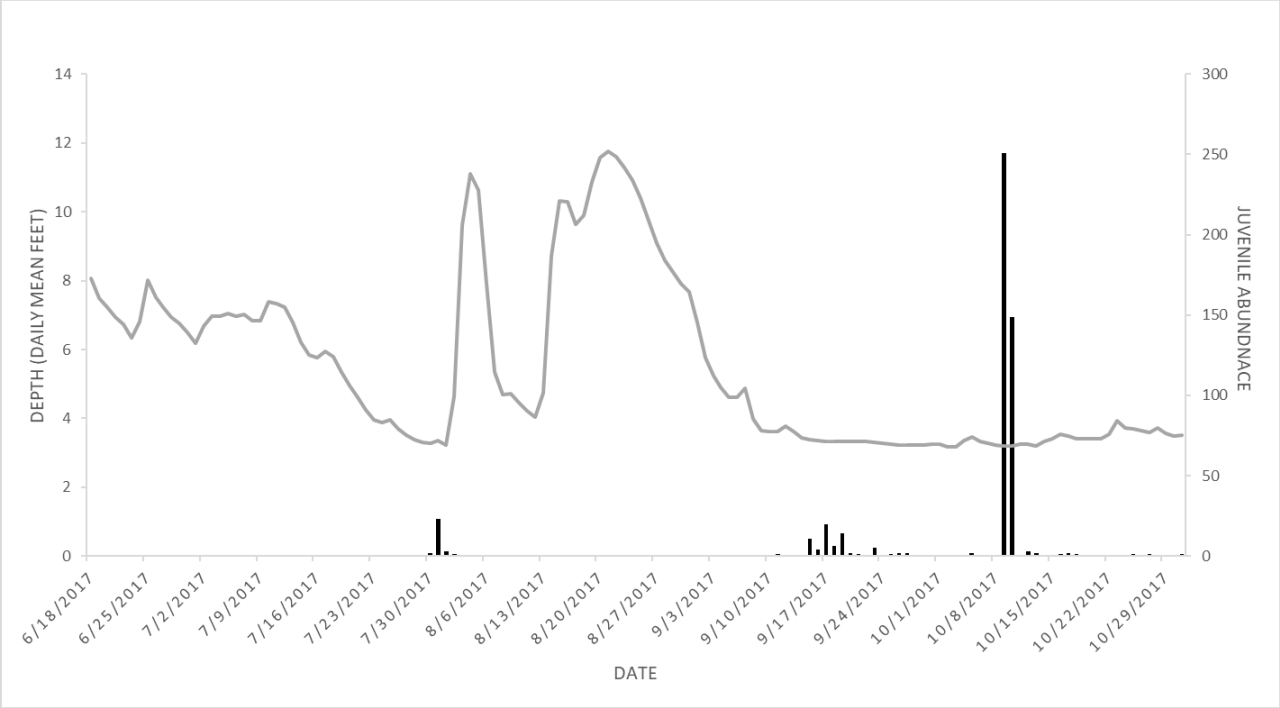


Figure 3. Relationship between depth (Texas Water Data Support Team, 2018) and juvenile abundance observed in lab. The y-axis on the left represents daily mean of depth near the sampling site in the Neches River, and the second y-axis in the right indicates juvenile abundance.

Table 3. Successfully sequenced juvenile *Pleurobema riddellii*, which were identified to species using National Center for Biotechnology Information database.

Identification Number	Date Collected	Fish Host	Mussel Species Identified with BLAST	Gene Used for Identification
166C	9-Oct	Blacktail shiner (*Cyprinella venusta*)	*Pleurobema riddellii*	ND1
199B	9-Oct	*C. venusta*	*P. riddellii*	ND1
430	10-Oct	*C. venusta*	*P. riddellii*	ND1
435	10-Oct	*C. venusta*	*P. riddellii*	ND1
442	10-Oct	*C. venusta*	*P. riddellii*	ND1
444	10-Oct	*C. venusta*	*P. riddellii*	ND1

Discussion

Six fully metamorphosed juvenile *P. riddellii* were obtained from *C. venusta.* This supports earlier suggestions of *P. riddellii* being a cyprinid specialist (Marshall et al., 2018) and follows the trend that other *Pleurobema* species also use cyprinids as hosts (Culp et al., 2009; Hove & Neves, 1994; Layzer et al., 2003). Contrary to Marshall et al.,

(2018), who found *P. riddellii* glochidia on *C. lutrensis* or *P. vigilax*, neither of those species produced juveniles in the current study. However, *C. lutrensis* and *P. vigilax* were much less common than *C. venusta* at our sample sites (Marshall et al., 2018). Because all *P. riddellii* juveniles were collected in early October, and because their time to metamorphize likely takes two to six weeks (Layzer et al., 2003; Culp et al., 2009; Hove & Neves, 1994; Weaver et al., 1991), results indicate that spawning time for *P. riddellii* is in late summer or early fall. Some data suggest that *P. riddellii* may also spawn in the Spring (Marshall et al., 2018), but the water was too high for sampling at that time in the current study so establishing spawning time is premature.

It is not surprising that *C. venusta* were found to be infected with *P. riddellii* juveniles, because these fish associate with the same habitat as *P. riddellii* adults. *Both C. venusta and P. riddellii* are found in swift runs with gravely substrate (Glen, 2017; Thomas et al., 2007). While the Red Shiner, *C. lutrensis,* also exhibit this same habitat preference and have been documented as the primary host to *P. riddellii* (Marshall et al., 2018), *C. lutrensis* was relatively less abundant than *C. venusta* in this study.

The maturation period from glochidia into juvenile for other mussels is estimated to be between two and six weeks (Culp et al., 2009; Hove & Neves, 1994), but looking back from the appearance of juveniles I could not detect a significant relationship in time to previous flow events or water depth and glochidia attachment. An in-lab study of *P. riddellii* would likely be necessary to accurately predict the timespan from encystment to juvenile metamorphosis, but this could lead to a better understanding of environmental factors which influence spawning.

Freshwater unionids are an important component of aquatic systems, and their preservation is vital to continuing the normal functioning of streams and rivers. These data provide federal and state agencies with the information to implement protective and management efforts for *P. riddellii* and indicate that *C. venusta* should be considered during their evaluation of its listing. A better understanding of life-history, migratory pattern, spawning, and immunological responses of *C. venusta* should be further studied to understand its relationship to *P. riddellii.* The data collected from this study provide further understanding of the freshwater ecology of East Texas rivers and gives future researchers a preliminary understanding of spawning pattern of *P. riddellii*.

Chapter 2: Population Structure and Life-History Characteristics of *Pleurobema riddellii*

Background

Energy spent toward growth versus other functions is a fundamental feature of an organism's life-history (Haag & Rypel, 2011). Mussel lifespan is negatively correlated with growth rate, and positively correlated with shell size and mass, which can explain some variation in lifespans (Haag & Rypel, 2011). Patterns of energy investment toward growth vary among mussel species. Several studies indicate that *Pleurobema* species exhibit slow, steady growth over a long lifespan (Haag, 2012; Haag & Rypel, 2011; Reátegui-zirena & Stewart, 2017).

Unionids also vary in sexual maturation and fecundity, for example *Lampsilis ornata* can produce about 48,000 glochidia at age 0, whereas *Quadrula pustulosa* are not sexually mature until age three and produce as few as 49 glochidia (Haag & Staton, 2003). Mussels that mature earlier have shorter lifespans, but high fecundity soon after maturation, whereas those exhibiting later maturation are less fecund in their first few years (Haag, 2012). Fecundity and reproductive effort vary among species. Some females can hold as few as 9,647 mature eggs within their gills (*Quadrula asperata*) while other species in similar conditions can hold up to 325,709 (*Amblema plicata*) (Haag & Staton, 2003). Mean annual fecundity varies among North American species nearly four orders of magnitude ranging from less than 2,000 to more than 8 million glochidia (Haag, 2012).

Genus *Pleurobema* falls within the tribe Pleurobemini. In this group reproductive effort and fecundity have been documented as being relatively low, for instance *Pleurobema collina* were found to produce only about 12,000 eggs per year (Haag, 2013;

Hove & Neves, 1994). Their life-history strategy is considered that of "equilibrium", which is characterized by a long lifespan, moderate to large adult size in relation to other unionids, short-term brooding, and low to moderate growth rates. While there has been little information collected on the life-history of *Pleurobema riddellii,* research on various other *Pleurobema* support this equilibrium strategy classification of short-term brooders, and having variable, but typically low fecundity (Culp et al., 2017; Hove & Neves, 1994; Layzer et al., 2016; Reátegui-zirena & Stewart, 2017; Weaver et al., 2016)

There is little information about the life-history characteristics of *Pleurobema riddellii*. Morphologically they are thick with an inflated, triangular to sub-quadrate shell, and exhibit a smooth outer shell with dark brown coloring and an umbo which rises above the hinge (Howells, 1996). They are sexually monomorphic, and are typically found in gravely mesohabitats within runs (Glen, 2017; Howells et al., 1996). Understanding reproductive characteristics and life-history traits is important for understanding of mussel biology and fundamentally being able to accurately manage species.

Analyzing gonadal fluid is a nonlethal, cost-efficient, and informative means of obtaining some unionid life-history and reproductive traits. Observable traits using this method include determining sex, age of sexual maturation, seasonal gamete production, gametogenesis, life-long fecundity, and sex ratios within a population. The technique of gonadal fluid extraction via hypodermic syringe was first used on a German population of the Pearly Mussel *Margaritifera margaritifera* during the 1980s. The method was initially intended to be a nonlethal means of distinguishing hermaphroditism (Bauer,

1987) but has shown efficacy in evaluating gamete production and sex determination, as it is both relatively accurate and noninvasive (Saha & Layzer, 2008; Tsakiris et al., 2016).

The technique of gonadal extraction is ideal for *Pleurobema riddellii* because they are relatively scarce, have state-listed status, and there is little information on their life-history and reproduction. Measuring gamete production can provide an estimation of fecundity and reproductive seasonality. Female gametes start as oogonium, developing into oocytes, and ultimately ovum. The objectives of this study were to monitor the stages of gametogenesis throughout the season to predict seasonal reproduction and lifelong fecundity, identify the threshold of sexual maturity, and understand aspects of the population structure of *P. riddellii* in east Texas.

Methods

Collecting wild mussels

Mussel beds with relatively high abundance of *P. riddellii* were chosen from localities previously sampled by personnel at the University of Texas at Tyler Biology Department (Ford et al., 2014). *P. riddellii* prefers mesohabits within gravely runs (Glen, 2017), so these mesohabitats were targeted. Sampling occurred in increments of approximately four weeks from March to December 2017. Designated mussel beds were reached via kayak or motorboat. Upon arrival to a sampling site, crew members tactilely searched for the *P. riddellii* and the first 8-10 individuals collected each period were processed. During each sampling period water flow, depth (Texas Water Data Support Team, 2018) and temperature were also recorded.

Processing procedure

Upon removal from the mussel bed *P. riddellii* mussels were processed in the following manner. They were first assigned an identification number, which was etched on their shells using a Dremel© tool so they were not resampled in the future. The mussels were then weighed to the nearest whole gram, measured with Vernier calipers to the nearest tenth of a millimeter (length, width and height), and aged by counting external annuli (Haag & Commens-Carson, 2008). Lastly, gonadal fluid (see procedure below) was extracted and the mussels were manually returned to the mussel bed. The same person completed all these processes.

Gonadal fluid extraction

Mussels were gently pried open with a modified stainless-steel nasal speculum with a narrow tip that was ground down. Mussels were open just slightly so that the tip of the thumb could be wedged between the open shell. While keeping the shell slightly agape, a 20-guage hypodermic needle was inserted at the lateral line on the anterior side of the visceral mass, just to the posterior side of the foot. The needle was gently moved back and forth within the viscera until gonadal fluid began to flow into the syringe. The plunger was pulled back gently until between 0.2mL and 0.5mL of gonadal fluid was extracted. Fluid was immediately put into a 1.5mL pre-labeled centrifuge tube, which contained 0.5mL 10% formalin buffer. The collected gonadal fluid was taken back to the University of Texas at Tyler laboratory for microscopy analysis.

Microscopy analysis

A subsample of formalin fixed gonadal fluid was put into a 0.5 mL microcentrifuge tube and one-part methylene blue to four-parts gonadal fluid solution

was added. Females were identified by the presence of viable or nonviable ovum, oocytes, or oogonium (Figure 4a), and males were identified by the presence of sperm (Figure 4b). Viable ova are characterized by an albuminous ring, which is lacking in nonviable ova, and sperm are recognizable by their flagella. The standards to compare these gametes were taken from previous publications, which delineated male and female gametes and developmental stages of gametes (Saha & Layzer, 2008). Individuals that exhibited neither female nor male gametes were reported as sexually unidentified. Females gamete concentrations were manually counted, and male gametes were counted using a hemocytometer (a counting chamber typically used to count single blood cells).

Statistical analyses

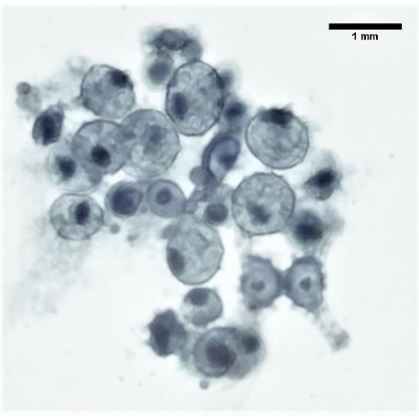
To understand the influences of environmental factors on *P. riddellii*, a Pearson's correlation was used to compare gametogenesis (production of gametes) with water flow, depth (Texas Water Data Support Team, 2018) and temperature. Additionally, a Pearson's correlation test was used to observe the relatedness of height, length, and width with age. Because some research indicates that there is a relationship between senescence and fecundity (Haag, 2012, 2013), a Pearson's correlation was also used to understand the relatedness of female age to fecundity (XLSTAT, 2017).

Results

Gonadal fluid analyses resulted in a total of 139 samples, 5 of which were positively identified as males, 97 were identified as females (52 producing only oognium or oocytes, and 45 produced nonviable or viable ova), and a remaining 37 were unknown because no gametes were identified (Figure 4a).

Gametogenesis of male *P. riddellii* ranged from mid-July to mid-August with the peak production occurring at 30 degrees Celsius. Concentrations ranged from 500,000 to approximately 20 million gametes per milliliter (n=5). Male gametes were flagellated and the average length and width were 4.2um and 1.96um respectively. The size of sexually mature males ranged from 37-50 mm and were estimated to be between ages nine and twelve according to external annuli (Figure 4b).

Female gametogenesis occurred from March to December with the peak production occurring at 25°C between early September and early October. Concentrations of gametes within a single female ranged from zero (but having clusters of oogonia and oocytes, which precede ova) up to 219,400 and 173,200 nonviable and viable ova, respectively (n=97). The size of sexually mature females (based on production of ova) ranged from 29-59 mm and was estimated to be between ages four and twelve according to external annuli (n=45). Among the entire female samples there was an average of 12,500 nonviable and viable ova per sexually mature female.

a) 

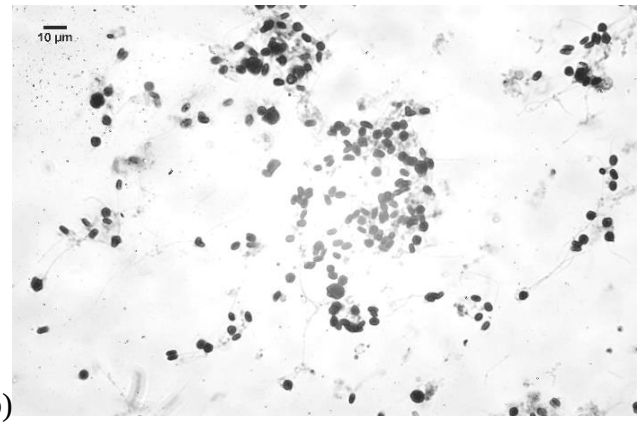
b) 

Figure 4. Photo a) a female *Pleurobema riddellii* nonviable ova and photo b) a sample of male sperm. This male had produced approximately 20 million sperm per milliliter.

Height, length, and width were associated with external annuli; the r^2 value for length was 0.51, the r^2 value of height was 0.59, and the r^2 value of width was 0.37

(Figure 5). Ova production of sexually mature females was not related to their external annuli ($r^2 < 0.01$) (Figure 6).

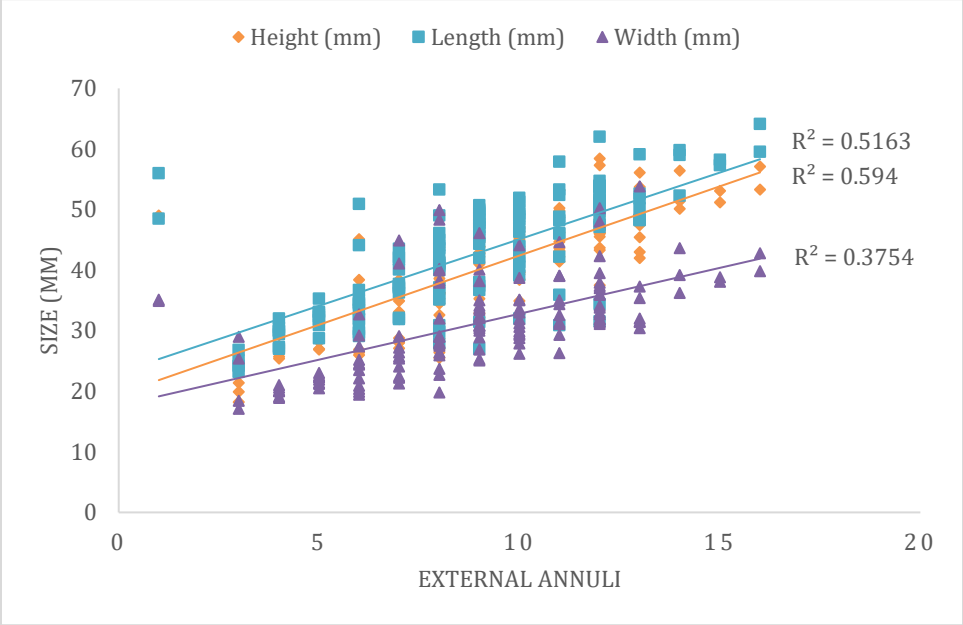


Figure 5. External annuli correlated with height, length and width of individual *P. riddellii.*

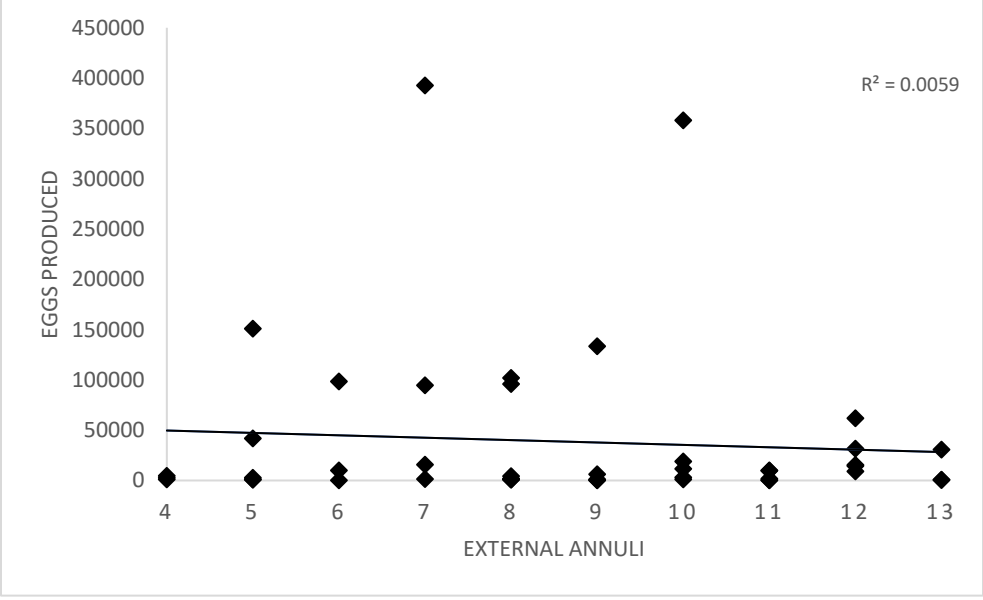


Figure 6. Correlation between ova production and age of female *P. riddellii* from March to December 2017.

Discussion

Overall fecundity in *P. riddellii* females collected for this study did not fluctuate between age groups. This indicates that once a female is sexually mature, age does not affect fecundity. The overall size of *P. riddellii* was moderate to large in relation to other species, and their growth increased slowly, but steadily over their lifespan, which supports the idea that this species like other Pleurobemini exhibits the equilibrium life-history strategy (Haag, 2012; Reátegui-zirena & Stewart, 2017).

The current study also found that mature females produced the most ova from early September to early October (25 degrees), and males produces the most sperm from mid-July to mid-August (30 degrees). However, analyses of gametogenesis was only observed for a 10-month span, not for January and February, although it seems unlikely that gametogenesis would occur in *P. riddellii* in winter. While this study did not observe brood-time, peak female gametogenesis was observed immediately preceding the approximate time of *P. riddellii* juveniles in the water (refer to Chapter 1). To definitively understand brooding periods future researchers should observe gravid females over extended periods, especially during late summer and early fall (refer to Chapter 1). Future research should focus on understanding the physiological and environmental factors which induce gametogenesis in both sexes. This study did provide further evidence for the efficacy of the non-lethal technique using a hypodermic syringe to obtain gonadal fluid for analyses and the insight it can provide into Unionid reproductive ecology (Saha & Layzer, 2008; Tsakiris et al., 2016; Bauer, 1987; Christian et al., 2000).

The low number of males found throughout this study was surprising and I suggest two possible explanations. There may simply be fewer males in the population.

Alternatively, because male gametes are much smaller than females, the sperm may have been overlooked during microscopy analysis. I did find several individuals which possessed no identifiable gametes that could have been undocumented males.

While *P. riddellii* do exhibit some characteristics of an equilibrium life-history strategy, further understanding of brooding periods is necessary to definitively classify them as such. Although preliminary these data provided information about relative seasonality of gametogenesis, sex ratios and size at first reproduction, and therefore insight into an east Texas population of *P. riddellii.* Such information can be applied to state and federal conservation and management efforts.

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Appendix. Data from other successfully sequenced juvenile mussels. Individual 28 was identified as having 95% identical nucleotide sequences as *Fusconaia askewi* and 96% identical to *F. lanenensis*. Individual number 95 was found to have 94% identical nucleotide sequences as both *F. lanenensis* and *F. askewi. Corbicula fluminea* are not within the order Unionidae and are not obligate parasites. It's unclear if the individuals found to be *Corbicula fluminea* were thought to be juveniles when collected, or if they were incidentally collected from environmental DNA.

Identification Number	Date Collected	Fish Host	Mussel Species Identified with BLAST	Gene Used for Identification
28	Aug-1	*Cyprinella venusta*	*Fusconaia lanenensis/askewi*	ND1
90	Sept-23	*Cyprinella venusta*	*Fusconaia askewi*	COI
92	Sept-23	*Cyprinella venusta*	*Fusconaia lanenensis*	ND1
95	Sept-26	*Cyprinella venusta*	*Fusconaia flava/askewi*	ND1
142	Oct-9	*Cyprinella venusta*	*Corbicula fluminea*	COI
142A	Oct-9	*Cyprinella venusta*	*Corbicula fluminea*	ND1
222B	Oct-9	*Cyprinella venusta*	*Corbicula fluminea*	ND1
346	Oct-9	*Cyprinella venusta*	*Corbicula fluminea*	COI
376	Oct-10	*Cyprinella venusta*	*Corbicula fluminea*	NDI